

# Journal of Visualized Experiments

## Semi-Minimal Invasive Method to Induce Myocardial Infarction in Rats and the Assessment of Cardiac Function by an Isolated Working Heart System --Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61033R2
Full Title:	Semi-Minimal Invasive Method to Induce Myocardial Infarction in Rats and the Assessment of Cardiac Function by an Isolated Working Heart System
Section/Category:	JoVE Medicine
Keywords:	Myocardial infarction ischemia/reperfusion remodeling working heart hemodynamics rat
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Vienna, 1090, Austria

**TITLE:**

**Semi-Minimal Invasive Method to Induce Myocardial Infarction in Rats and the Assessment of Cardiac Function by an Isolated Working Heart System**

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**KEYWORDS:**

myocardial infarction, ischemia/reperfusion, remodeling, working heart, hemodynamics, rat

**SUMMARY:**

This article presents an efficient method to perform myocardial ischemia and subsequent chronic reperfusion in rats using a minimally invasive approach. In addition, left ventricular hemodynamic function of rats is assessed by echocardiography and isolated working heart methods.

**ABSTRACT:**

Myocardial infarction (MI) remains the main contributor to morbidity and mortality worldwide. Therefore, research on this topic is mandatory. An easily and highly reproducible MI induction procedure is required to obtain further insight and better understanding of the underlying pathological changes. This procedure can also be used to evaluate the effects or potency of new and promising treatments (as drugs or interventions) in acute MI, subsequent remodeling and heart failure (HF). After intubation and pre-operative preparation of the animal, an anesthetic protocol with isoflurane was performed, and the surgical procedure was performed quickly.

Using a minimally invasive approach, the left anterior descending artery (LAD) was located and occluded by a ligature. The occlusion can be performed acutely for subsequent reperfusion ischemia/reperfusion injury. Alternatively, the vessel can be ligated permanently to investigate the development of chronic MI, remodeling or HF. Despite common pitfalls, the drop-out rates are minimal. Various treatments such as remote ischemic conditioning can be examined for their cardioprotective potential pre-, peri- and post-operatively. The post-operative recovery was quick as the anesthesia was precisely controlled and the duration of the operation was short. Post-operative analgesia was administered for three days. The minimally invasive procedure reduces the risk of infection and inflammation. Furthermore, it facilitates rapid recovery. The “working heart” measurements were performed ex vivo and enabled precise control of preload, afterload and flow. This procedure requires specific equipment and training for adequate performance. This manuscript provides a detailed step-by-step introduction for conducting the measurements.

## **INTRODUCTION:**

Although incidence is continuously decreasing, acute myocardial infarction (MI) is still the major contributor to morbidity and mortality worldwide<sup>1</sup>. There are restrictions on evaluating the efficacy of potential treatments as drugs or surgical procedures which prevent and treat acute MI. Before their effects can be examined in humans, these treatments must be tested for risks in advance, including in vivo examinations in animals. There is no better opportunity to study a pathology than under in vivo conditions. Therefore, MI induction in rats or mice and even large animal models (pigs or sheep) permits the investigation of short- (acute) and long-term (chronic) changes due to ischemia in the coronary arteries and the surrounding myocardium, as well as systemic changes due to impaired cardiac function. Infarct size was previously the main objective, but more recently subsequent cardiac remodelling processes in acute MI or ischemia/reperfusion injury as well as in consecutive heart failure (HF) have become of great interest. Therefore, a comparable and easily reproducible method is required to reach consistent outcomes.

While the use of cryo-ablation to gain MI has been reported<sup>2</sup>, our method builds on other studies in which investigators occlude the left anterior descending artery (LAD) by a single stitch ligation. In comparison to (hemi-)sternotomy procedures, the minimally invasive approach which will be presented in this article, permits a faster post-operative recovery and markedly reduces the operation time. A common step of other surgical procedures is the lift-out of the heart from the thorax to perform the heart stitch<sup>3</sup>. The approach of this method renders this step unnecessary. Depending on the protocol, two different procedures can be performed: a temporary occlusion using a tourniquet to induce ischemia/reperfusion over a defined time; or a permanent occlusion of the artery by fixing the ligature. The success of occlusion can be evaluated with electrocardiogram (ECG) and the macroscopic changes in the left ventricle (LV) as well as its paling.

Another important step prior to the surgery is intubation. While in most cases, intubation is performed via tracheotomy or via oral insertion of the tube under vision by a skin incision at the throat, this protocol describes the endotracheal intubation of the anesthetized animal which reduces breathing difficulties or infections post-operatively<sup>4,5</sup>. To avoid post-operative

complications, air is removed from the thorax via a syringe before closing the chest.

The second task of this article is the evaluation of hemodynamic function via an isolated working heart experimental model, which is how it is used in other projects within our institute<sup>6,7</sup>. While, echocardiography, cardiac magnetic resonance imaging (MRI) and invasive quantification of pressure-volume loops are well-known and widely used methods to assess cardiac function in vivo, they are known to have some limitations. Invasive approaches, such as the use of catheters to examine the global function or specific parameters of the heart, are commonly used and represent the gold standard of cardiac measurements. In contrast, the ex vivo working heart apparatus is rarely used because of its complexity and cost. There are many important aspects, from the mixture of the perfusate to the adequate cannulation of the heart, which are crucial for successful evaluation. The isolated working heart apparatus was firstly described by Oskar Langendorff in 1897<sup>8</sup> and has been modified over the recent decades<sup>9</sup>. Today, there are two models used: the Langendorff (LD) mode and the working heart (WH) mode. In our study, the LD mode is used to acclimatize the heart to its new environment (about 15 min). In this mode, the heart is cannulated via the aorta and the coronary arteries are perfused anterogradely, adequately supplying the myocardium. In the LD mode, the heart is not performing any pressure-volume work. By contrast, in the WH mode, the left atrium is cannulated via a pulmonary vein, through which the perfusate enters the left atrium. The heart then pumps this perfusate physiologically against a predefined afterload. By increasing the afterload over time, cardiac function can be continuously measured. Parameters such as coronary flow, cardiac output (CO), stroke volume (SV) and work, atrial flow and LV systolic and diastolic pressures can be measured. The impact of various treatments directly and solely on the heart can be investigated<sup>6,10</sup>. A review by Liao and Podesser<sup>9</sup> presented the widespread use of this method in the evaluation of pharmacological effects on cardiac function and metabolism as well as in the exploration of various diseases, such as MI, HF, obesity and.

In summary, this protocol presents a reproducible method to perform MI or myocardial ischemia/reperfusion (MIR) injury in vivo. In addition, it permits the characterization of LV (dys-)function on an isolated rat heart after MI. This protocol presents a unique combination of treatment and analysis.

## **PROTOCOL:**

The experimental protocol which delivered the results described in this article has been approved by the regional Ethics Committee for Laboratory Animal Experiments at the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Research (BMWFW-66.009/0023-WF/V/3b/2016). All experiments conform with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

NOTE: 10–12-week-old male Sprague Dawley rats of 250–300 g body weight (BW) are used. As the following procedures and treatments are performed in a sterile environment of an operating room (OR), wear scrubs, gloves, facemasks and hoods when handling animals. Before entering

the OR, ensure that hands are washed and disinfected. If the intention is to operate on several animals in a surgical session, either wash and disinfect, or autoclave the instruments in between operations. These hygienic guidelines are valid for all procedures presented in the protocol section.

## **1. Preoperative preparation and anaesthesia**

1.1. Initiate preoperative anaesthesia by injecting a mixture of xylazine (4 mg/kg BW) and ketamine (100 mg/kg BW) intraperitoneally.

1.2. Intubate the rats with a 14 G tube and volume-controlled ventilation with a mixture of O<sub>2</sub>, air and isoflurane (1–2.5%) at 75–85 strokes/min, 100 mL/stroke/BW (**Figure 1A**).

1.3. Place the rats on a heated operating table in a supine position and fix the forelimbs with tape (**Figure 1B**).

1.4. Measure rectal temperature with a probe.

NOTE: It should be maintained between 37.5–38.5 °C.

1.5. Shave the thorax and clean the operating area with antiseptic povidone iodine solution. Apply eye ointment to the rat to prevent drying of the eyes.

1.6. Administer intraoperative analgesia by injecting piritramide (0.1 mL/kg BW) intraperitoneally.

1.7. Place ECG probes subcutaneously in the extremities of the animal.

1.8. Check tail and toe reflexes prior to initiating the surgical procedure.

## **2. Surgical procedure—induction of myocardial ischemia**

2.1. Perform skin incision using a scalpel. Ensure to start 2 mm parasternal on the left thorax at the level of the 3rd intercostal space and continue to the anterior axillary line at the level of the 5th intercostal space (**Figure 1C**).

2.2. Replace the superficial muscles gently to make the ribs visible (**Figure 1D**).

2.3. In the case of minor bleeding, use a cauter to obliterate or to disconnect the surrounding tissue.

2.4. Perform the thoracotomy at the level of the 4th intercostal space and insert a retractor to gain visibility of the heart and the lung (**Figure 1E**). Carefully open the pleura and the pericardium to avoid bleeding.

2.5. Temporarily occlude the LAD using a tourniquet to induce ischemia/reperfusion (MIR) over a defined time; or permanently (MI) occlude it by making 6–7 knots using a 6-0 suture to close the ligation (**Figure 1F,G**).

NOTE: The right spot for occlusion of the LAD is located about 2–3 mm beneath the left auricle on the left lateral margin of the heart. Successful occlusion is associated with ECG changes (ST-segment elevation) and macroscopic changes in the LV as paling.

2.6. In the case of the ischaemia/reperfusion model, reopen the LAD by removal of the tourniquet after 30 min of occlusion.

2.7. Close the thorax with three single button sutures using a 4-0 suture and a single monofilament (**Figure 1H**). Prior to tightening the last suture, remove any residual air from the thorax with a 5 mL syringe to prevent a pneumothorax (**Figure 1I**).

2.8. Reposition the muscles and the volatile anaesthesia.

2.9. Suture the skin with a continuous suture using a 4-0 suture (**Figure 1J**).

2.10. Administer an antiseptic spray to protect against infections and biting of the suture by rats.

### 3. Postoperative treatment and exclusion criteria

3.1. Keep the rats on the heating table until they wake up. Extubate the rats as soon as they commence breathing spontaneously.

3.2. Put the extubated rats in a cage under a heating lamp to prevent them from cooling.

3.3. Return rats to the animal houses under standardized conditions when they commence behaving normally again.

3.4. Add 2 ampules of piritramide and 30 mL of 5% glucose to 250 mL of water for post-operative analgesia for three days.

3.5. Check the fitness and behavior of rats with the checklist and exclusion criteria (**Table 1**). Observe the animals twice a day for the following week, then twice a week.

[Place **Table 1** here]

NOTE: In accordance with international standards, present any suffering animals, or animals that gain up to 6 points in the evaluation with the checklist, to veterinarians to make therapy-related decisions. Any animals that gain 7 or more points must be immediately sacrificed with an overdose of ketamine and xylazine.

#### 4. Echocardiography measurements

NOTE: Echocardiography is usually performed twice, prior to the induction of MI and before the organs are harvested.

4.1. Inject rats with a mixture of xylazine (4 mg/kg BW) and ketamine (100 mg/kg BW) intraperitoneally.

4.2. Place the rats in a supine position on a heating tray. Apply echo gel to the chest, which helps the ultrasound waves travel better and reduces signal interferences.

4.3. Obtain parasternal short axis views of the LV cavity at the level of the papillary muscle.

4.4. Perform M-mode echocardiography in order to measure left ventricular ejection fraction and morphology.

#### 5. Organ harvesting (without working heart)

5.1. Administer xylazine (4 mg/kg BW) and ketamine (100 mg/kg BW) intraperitoneally prior to organ harvesting. Ensure that the reflexes are negative.

NOTE: No intubation is required as the procedure does not last longer than 1 min.

5.2. Use a scalpel to make a skin incision under the xiphoid and extend it parallelly to the ribs on both sides using scissors.

5.3. Cut the ribs in the frontal axillary line and grab the xiphoid to lift the chest up (Figure 2A).

5.4. Remove anatomical or fibrotic tissue adhesions by carefully rupturing the tissue with two pairs of forceps.

5.5. Take blood samples (for blood gas evaluation or molecular analyses) from the vena cava inferior with a 5 mL syringe.

5.6. Perform the excision of the whole heart at the inlet and outlet level (Figure 2B). If necessary, proceed with working heart evaluation as described in section 6.

5.7. Harvest organs, shock frost them in liquid nitrogen and store in -80 °C for further molecular analyses, or in formaldehyde for histological purposes.

#### 6. Ex vivo hemodynamic measurements via a working heart system

NOTE: The general setup and the components of the apparatus has been previously described<sup>11</sup>.

The following protocol describes the handling of the animal's heart and the necessary steps to evaluate LV function.

6.1. Anesthetize rats as described in step 5.1 and inject 200 IU of heparin intravenously (femoral vein).

6.2. Open the thorax via an incision beneath the costal arch with a scalpel and extend it to both anterior axillary lines with scissors and elevate the sternum.

6.3. Cut the great vessels near their outlet or inlet to the heart to excise it (**Figure 2B**).

6.4. Immerse the heart in ice-cold Krebs-Henseleit buffer and mount it on the erythrocyte-perfused isolated heart system via cannulating the aorta (**Figure 3A**).

6.5. Start with the LD mode with a constant afterload of 60 mmHg (stabilization period).

6.6. After 15 min of LD mode, switch to the WH mode. Therefore, cannulate the left atrium via a pulmonary vein (**Figure 3B**). Then, change the flow direction in the system by opening the clip that occludes the atrial cannula. This results in a perfusion of the left atrium and a physiological blood flow in the left heart<sup>11</sup>.

6.7. Record hemodynamic measurements for 20 min in the WH mode.

6.8. Collect blood drops of the coronaries with a 2 mL syringe to measure coronary flow (CF, mL/min) every 5 min.

NOTE: CF is measured as the difference between left atrial flow (LAF) and aortic flow (AF).

6.9. Perform continuous measurements of LAF (equivalent to cardiac output) and AF with a flow probe.

NOTE: The probe is inserted via the WH apparatus into the LV. All data are continuously registered.

6.10. If the ongoing protocol requests, insert a high-fidelity catheter retrogradely via the aortic valve into the LV and measure the left ventricle systolic pressure (LVSP).

6.11. To assess the pressure–volume work performed per minute, calculate stroke volume as cardiac output divided by heart rate.

6.12. Calculate external heart work (EHW) according to the following formula:  $CO \times LVSP$  (g x m/min) normalized to heart weight.



## REPRESENTATIVE RESULTS:

The following results have been published in Pilz et al.<sup>6</sup>. With this precise surgical procedure, the cardioprotective effect of remote ischemic preconditioning (RIPerc) can be investigated. This is a potential new treatment for patients suffering from acute MI or MIR and subsequent ventricular remodeling, which in many cases leads to consecutive HF. Mimicking the pathophysiological changes of MI/MIR is an obligatory step in the evaluation of treatments as in vitro or ex vivo studies do not provide the physiological environment. In this protocol, the animals were subjected to 30 min of LAD occlusion followed by reperfusion (i.e., MIR).

To prove the reproducibility of the procedure, histological cuts and stains were performed (**Figure 4A**). It was clear that the fibrotic scar in MIR+RIPerc treated animals was comparable with the scar formation of the Sham animals while the comparison of fibrosis between Sham and MIR groups was significant (**Figure 4B**). Additionally, MIR+RIPerc treated animals showed significantly reduced fibrosis compared to MIR-treated animals. However, the representative histological images clarify the potency of this surgical procedure as the infarction is explicitly sustained in the MIR group (**Figure 4A**). Using in vivo echography, ejection fraction, LV end-diastolic and end-systolic diameters (LVEDD and LVESD) were measured and showed significantly reduced cardiac function due to MIR treatment while hemodynamic parameters were preserved by RIPerc (**Figure 4C–F**). Ex vivo hemodynamic data exhibited the effectiveness of the procedure as the MIR group showed significant decreases in LVSP, cardiac output (CO), stroke volume (SV) as well as external heart work (EHW) (**Figure 5A–G**).

A literature search about this surgical procedure reported no negative or unsatisfying comments and results when it was adequately performed. Nevertheless, pitfalls mentioned in the introduction and the discussion need to be prevented and training is mandatory to acquire a stable level of performance and to obtain comparable results.

## FIGURE AND TABLE LEGENDS:

**Figure 1: Preoperative preparation and surgical procedure.** (A) Intubation of the animal using a 14 G tube. (B) Supine positioning and disinfection of the surgical field. (C) Skin incision (2 mm parasternal on the left thorax at the level of the 3rd intercostal space). The incision must reach the anterior axillary line at the level of the 5th intercostal space. (D) Displace the muscles to make the ribs visible. (E) Opening of the thorax. (F) Permanent occlusion of LAD using 6–7 knots. (G) Transient occlusion of LAD using a tourniquet. (H) Closure of the chest after myocardial ischemia and reperfusion by placing three single-knot sutures around the ribs. (I) Proper closing of the thorax. Use a 5 mL syringe to remove any residual air from the thorax before fixing the last knot tightly. This is integral to prevent a pneumothorax. (J) Skin suture.

**Figure 2: Organ harvesting.** (A) Open the chest with sub-xiphoidal cuts in both mid-axillary lines. Further cuts through the ribs are performed to facilitate lifting of the sternum. (B) Excision of the heart.

**Figure 3: Isolated heart apparatus.** (A) Langendorff mode. The heart is mounted to the WH

apparatus via cannulation of the aorta. (B) Working heart mode. The system can be switched to the WH model to evaluate cardiac function by cannulating the left atrium.

**Figure 4: Effect of remote ischemic conditioning on scar formation, left ventricular function and remodeling.** (A) Histological LV slices harvested on day 14 post-myocardial reperfusion. (B) Quantified results of fibrosis in bar graphs. (C) Representative M-mode echocardiograms. (D) Ejection fraction (EF) quantified in bar graphs. (E) LV end-systolic diameter (LVESD) quantified in bar graphs. (F) LV end-diastolic diameter (LVEDD) quantified in bar graphs. MIR, myocardial ischemia-reperfusion; RIPerc, remote ischemic preconditioning. Data are expressed as mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Reprinted from Pilz et al.<sup>6</sup> with permission from Elsevier.

**Figure 5: Effect of RIPerc on LV hemodynamic function.** (A) LV systolic pressure (LVSP), (B) cardiac output (CO), and (C) stroke volume (SV) results were obtained from the isolated working heart on day 14 post-myocardial reperfusion. (D) CO is depicted as a function of afterload; (E) external heart-work as function of afterload, quantified results in bar graph (E and G). Data is expressed as mean  $\pm$  SEM and  $n = 4-7$  per group. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . MIR, myocardial ischemia/reperfusion; RIPerc, remote ischemic preconditioning; EHW, external heart work; SV, stroke volume; AUC, area under the curve. Reprinted from Pilz et al.<sup>6</sup> with permission from Elsevier.

**Table 1: Checklist and exclusion criteria.** This table contains the examinations that must be observed and the corresponding score. Accordingly, the post-operative treatment of the animal must be adapted, or a veterinarian must be consulted.

## DISCUSSION:

Adverse remodeling post-MI is considered to be a key mechanism in the development of heart failure. Therefore, to ensure the continuity of cardiovascular research, experimental procedures and techniques should be reproducible. A comprehensible and clearly defined experimental protocol is a fundamental element of reproducibility. Reproducibility refers to results that can be repeated by multiple scientists and are validated across laboratories. This study aimed to present a semi-minimally invasive method to induce chronic or re-perfused MI and assess the cardiac hemodynamic function in rats.

These results and further published data show the high potency of this surgical method and its importance in research on MI, remodeling and HF. While ischemia/reperfusion injury can be used to understand the changes in MI with subsequent reperfusion, permanent occlusion allows further understanding of the short- and long-term remodeling processes of the myocardium. Other surgical approaches cause more tissue damage and animals show higher risks of developing infections and pneumothorax, resulting in higher drop-out rates. In contrast, this procedure is aimed to reduce mortality by specific improvements in the setup and handling. Additionally, they show variations in fibrotic scar expansion due to unstable LAD occlusion.

Our protocol provides an easy method for intubation, which is one of the most critical steps of the whole procedure. In contrast to several other publications<sup>12</sup>, tracheotomy is not performed

in our procedure. This enhances awakening and rehabilitation of the animals post-operatively, leading to the development of the pathophysiological changes which are intended by this surgical procedure before the animals undergo post-operative measurements. Obviously, if it is a non-survival protocol, tracheotomy is performed under vision and is thus easier to perform. Additionally, closing of the tracheotomy in a survival-protocol is not applicable. If the thorax is opened, it is mandatory to ventilate the lung to prevent collapse. Therefore, the rats are intubated prior to the surgical procedure. The minimally invasive approach does not cut the ribs or sternum thus maintaining the compactness and stability of the thorax. Consequently, the animals' recovery is improved, and the risk of spontaneous pneumothorax or bleeding is relatively low.

As aforementioned, while the intubation is of clear advantage, it is difficult to perform and may cause a higher drop-out rate at the beginning of the experiments. This problem can be mitigated with training and some anatomical knowledge. It is important to insert the tube at the right angle and stretch the animal's body until the light shines through the vocal lips after which the tube can be gently pushed forward. Take care to not harm the vocal lips as this can cause swelling, subsequent occlusion of the glottis and suffocation.

It is also important that the LAD is ligated correctly. The small surgical window, fast beating heart, and ventilated lung (avoid touching it as much as possible as every contact may result in bleeding in the lung) render the vessel not clearly visible. Therefore, anatomical knowledge is indispensable. The left auricle is indispensable to help standardize both the area at risk and to position the ligation around the LAD. The stitch needs to be performed intramurally, not transmurally in the LV as this may cause a reduction of the LV chamber diameter and volume which is not due to the pathological processes. Successful occlusion is associated with cyanosis of the myocardial area at risk and elevation of ST-segment on ECG. The main limitation of this procedure is the correct positioning of the suture. To achieve comparable results, the stitches must be at the same level and need to use similar amounts of tissue. This requires a high level of training and the different weights of the animals must be considered. Another point to consider is the adequate removal of the pneumothorax prior to closure of the intercostal space. If this is not precisely performed, the animals will exhibit difficulties in breathing as inflation of the left lung will be hindered by a pneumothorax. As aforementioned, this can be mitigated by using a syringe to remove any residual air from the thorax.

Currently, this MI procedure is a commonly used method which guarantees comparable results and a high survival rate if the critical steps are performed with high precision. Future projects on various treatments, devices or drugs in MI, HF or cardiac remodeling can be evaluated by performing this minimally invasive technique.

The WH measurements are, as aforementioned, not commonly used as its maintenance and handling requires specific equipment and knowledge. To acquire representative and comparable data, pitfalls must be avoided. The most critical steps are the mounting of the heart and switching from the LD model to the WH mode. If the heart is not excised adequately, the mounting may be difficult as sufficient aortic tissue length is required to fix the heart to the apparatus. Soon after

connecting to the LD mode, the heart frequency may decrease due to the washing in cold buffer, the disconnection of its physiological stimuli in the body or the reperfusion with blood from another species by the apparatus. In such cases, a pacemaker must be applied to both restore and preserve the physiological frequency. This ensures comparable results in all animals. As the blood volume within the apparatus is a multiple of the physiological volume in rats, bovine red blood cells in a Krebs-Henseleit buffer-based suspension are used.

The switch from the LD mode to the WH mode is synonymous to a switch from passive to active heart work. The LD mode is used to accustom the heart to its new environment. In the WH mode, the heart must perform its physiological ejection functions. Therefore, a short adaption phase to the new circumstances is required before the evaluation by increasing the afterload.

Another critical step which is commonly forgotten is the adequate preparation and maintenance of the apparatus and the perfusate. The precise volume of each compound must be mixed and the temperature within the system must be controlled and adjusted. Nevertheless, the WH is an elegant method to assess cardiac output, stroke volume, left ventricular systolic pressure and coronary flow simultaneously.

This highly reproducible procedure to induce MI and the representing data acquired by the WH apparatus are proving their capability themselves. The semi-minimally invasive approach, the level of LAD occlusion and intubation method facilitate fast recovery and low variability in infarct size. Additionally, cardiac function analysis in isolated working hearts provide valuable hemodynamic results.

#### **ACKNOWLEDGMENTS:**

The authors thank the operation theater team and technicians of the Center for Biomedical Research for their contribution, technical assistance, valuable input and advice. The projects are funded by the Ludwig Boltzmann Institute, Cluster for Cardiovascular Research (REM project).

#### **DISCLOSURES:**

The authors have nothing to disclose.

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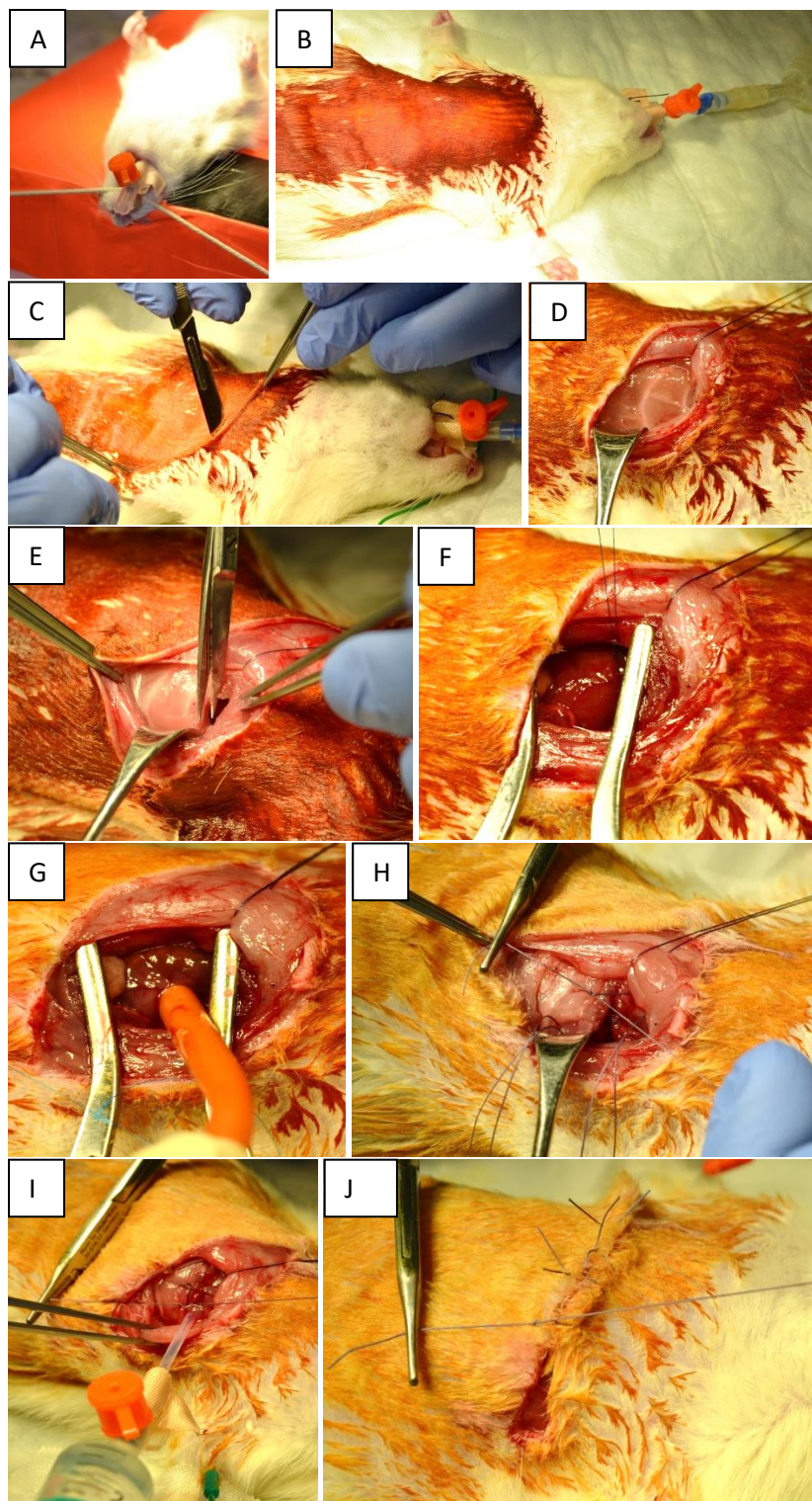
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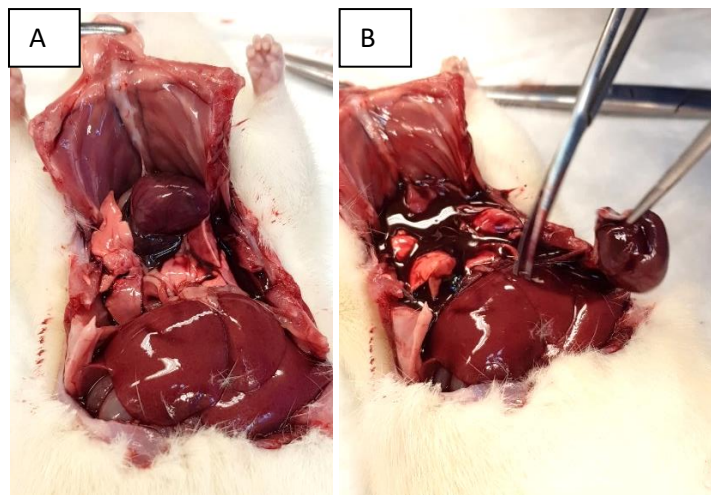
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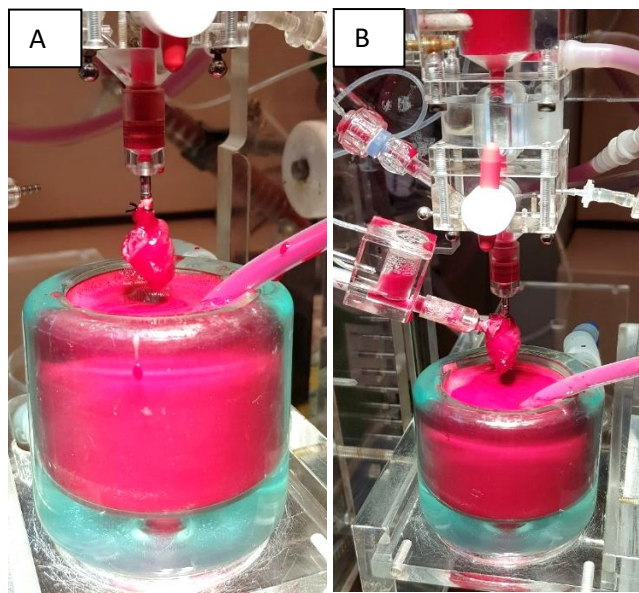
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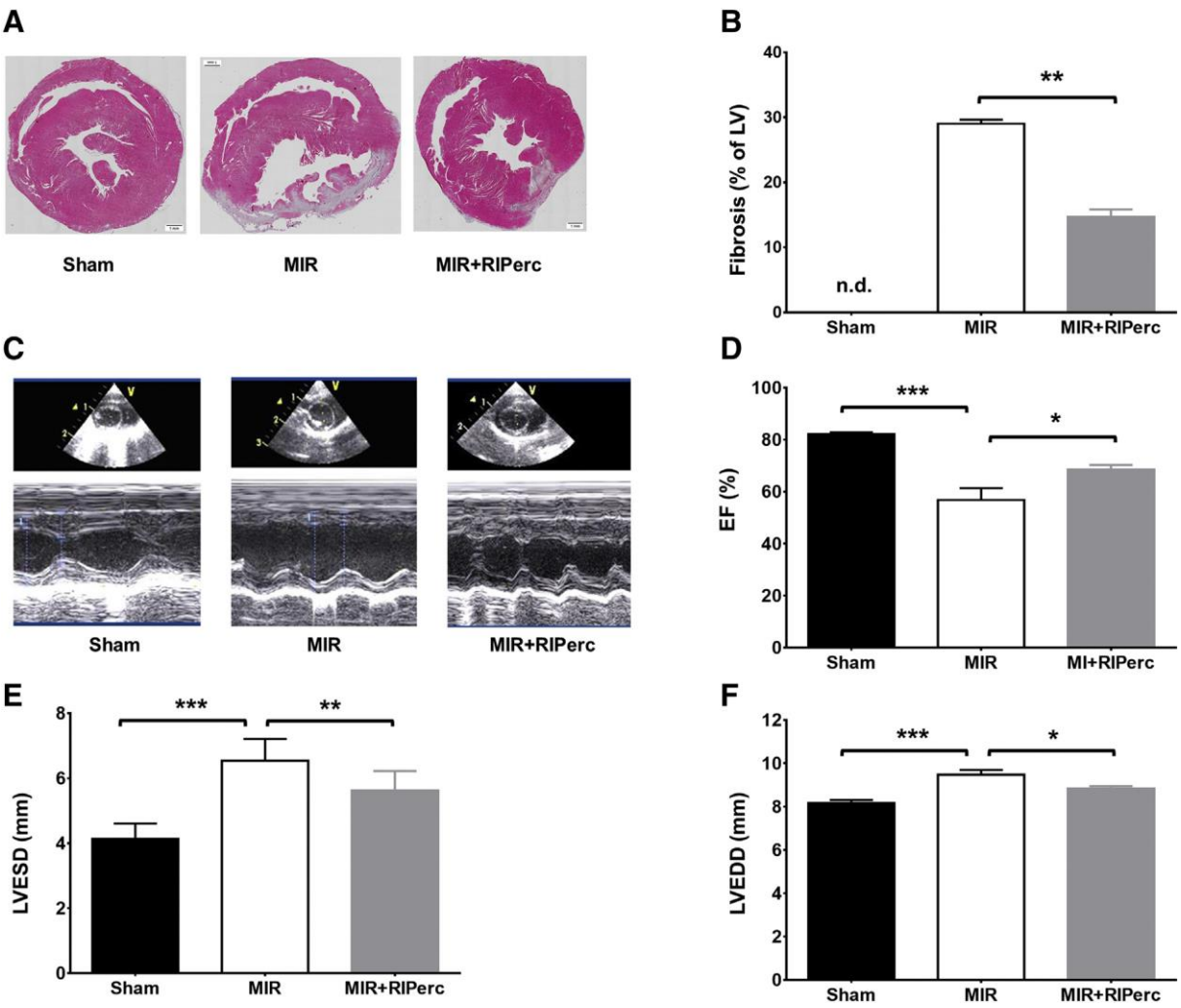


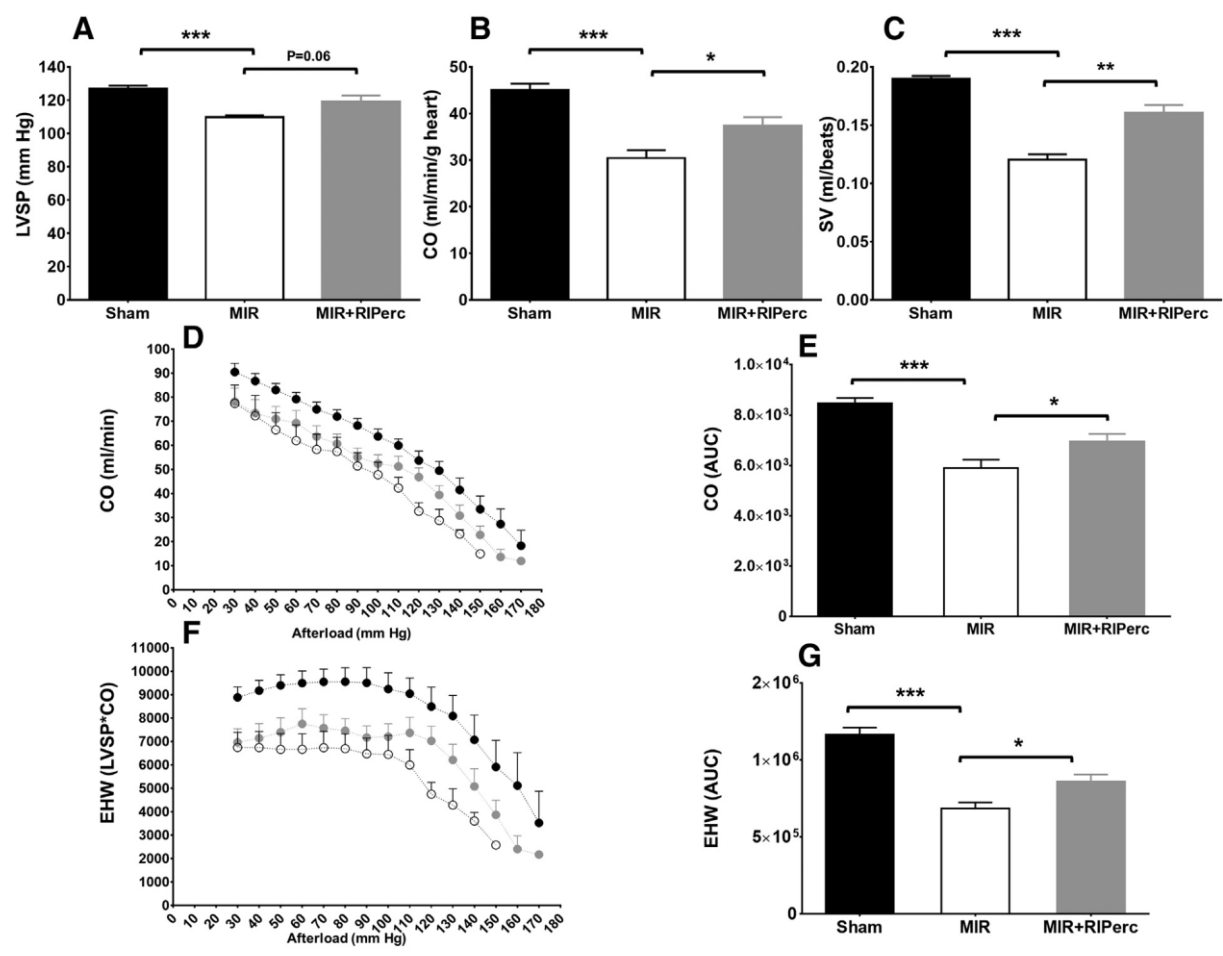












Examination	Observation	Score
Body weight	stable	0
	10% loss	4
	15% loss for 48 h	7
	18% loss	7
	normal (coat flat and shiny)	0
External appearance	piloerection	1
	haematoma	2
	skin wounds/cuts/bite marks	2
	severely reduced grooming	4
	(orifices unclean/clotted or moist)	7
	severe skin irritations or wounds	7
	hunched posture >2 h	7
	significant abdominal distension (ascites)	7
Behavior	normal (sleeping, curious, social contacts, reaction when touched)	0
	unusual behaviour, e.g. impaired activity	2
	self-isolation, pronounced hyperactivity or stereotypia	4
	lethargia for <6 h	4
	lethargia for 6 h to 8 h	7
	apathia >8 h	7
	stereotypia uninterrupted for >10 min. and still persisting after 2 h	7
	signs of pain when touched	7
	automutilation	7
Digestion	normal	0
	diarrhea (soft feces)	3
	diarrhea for 72 h or watery	7
	bloody stool	7

Name of Material/Equipment	Company
----------------------------	---------

ANAESTHESIA & ANALGESIA	
Isoflurane	Zoetis
Ketamine	Dr. E. Gräub AG
Piritramide	Hameln-Pharma Plus GmbH
Xylazine	Bayer

INTUBATION	
Air	
Oxygen (pure)	
Ventilation machine	Hugo Sachs Electronics
14-gauge tube	Dickinson and Company

PREPARATION	
Anti-septic povidine iodine solution	Mundipharma
Eye ointment	Fresenius Kabi Austria
Shaver	

SURGICAL INSTRUMENTS	
Anatomical forceps	Martin
Anatomical forceps small	Martin
Anatomical forceps thin	Odelga
Cautery Fine Tip	High Temp
Cup (small, for liquids)	Martin
Mensur	MTI
Mosquito clamps	MTI
Needleholder short	Martin
Needleholder thin	Martin
Round hook	
Scalpell size 3	Swann Morton
Scissors for tissue preparation	Aesculap

Sharp scissors	MTI
Small retractor	Alm
Surcigal forceps	Martin
Surgical scissors	

<b>SUTURES</b>	
PermaHand Silk 4-0	Johnson & Johnson Medical Products GmbH
Vicryl 4-0	Johnson & Johnson Medical Products GmbH
Vicryl 6-0	Johnson & Johnson Medical Products GmbH

<b>COMPUTER PROGRAMS &amp; APPARATUS</b>	
Labchart 7 Pro	ADInstruments
PowerLab System	ADInstruments

<b>EX VIVO HEMODYNAMICS</b>	
Flowmeter Narcomatic RT-500	Narco Bio-Systems
Isolated heart apparatus	Hugo Sachs Electronics
Labchart 7 Pro	ADInstruments GmbH
Millar SPR-407	Millar Instruments Inc.
Needle electrodes via Animal bio Amp	ADInstruments GmbH
Physiological Pressure Transducer (MLT844) with Clip-on BP Domes	ADInstruments GmbH
PowerLab System	ADInstruments GmbH

Catalog Number	Comments/Description
----------------	----------------------

TU061219 / 8-00487	
	100 mg/kg of bodyweight
	2 ampulles with 30 ml of Glucose 5% in 250ml water
	4 mg/kg of bodyweight

UGO Basile S.R.L.	Respirator
	BD Venflon

	Betaisodona solution
	Oleovital with Vitamin A + Dexpanthenol

12-272-15	
24-386-16	
RU4042-15	
	bvi-Accu-Temp
56-231/11	
29-260/25	
05-055/12	
20-658-14	
BT-190	
No.10, 0301	
BC259R	

01-010/10	
AM.416.10	
12-321-13	

K891H	
JV2024	single monofil suture
V301G	polyethylene suture

v7.3.2	Labchart Software
Powerlab 8/30	

	flow probe
v7.3.2	Labchart Software
840-4079	high-fidelity MicroTip catheter
MLA1203	
MLT844	
Powerlab 8/30	

## **Revision No.1 of manuscript JoVE61033, Response to reviewers' and editor's comments**

### **Editorial comments:**

We thank the editor for their valuable comments. Please find our responses below.

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.**

Thank you for this comment. The manuscript has been proofread carefully by the authors and a native speaker. Spelling and grammar mistakes have been corrected by a native speaker.

**2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."**

This has been modified and changed accordingly. The relevant document has been submitted.

**3. Please revise lines 282-285 to avoid textual overlap with previously published work.**

The changes have been made accordingly in the revised version of the manuscript (page 9, lines 346-349).

**4. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.**

We thank the reviewer for this comment. It is stated in the methods section that the animal experiments were performed according to the Guide and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23,



revised 1996). Please see the revised version of the manuscript (page 6, lines 237-242).

**5. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.**

The changes have been made accordingly in the revised version of the manuscript.

**6. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.**

We have rephrased the protocol section in the revised version of the manuscript (pages 3-4, lines 116-122). We hope that the paragraph about safety procedures now provides clear instructions.

**7. For each protocol step, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.**

The changes have been made in the revised version of the manuscript accordingly.

**8. Please present the content in Figure 1 in a table (Table 1) and upload the table to your Editorial Manager account as an .xlsx file.**

The content in Figure 1 has been translated into Table 1 which has been included in the revised version of the manuscript.

**9. Please remove the titles and figure legends from the uploaded figures. Please include all the figure Legends together at the end of the Representative Results in the manuscript text.**

The changes have been made accordingly in the revised version of the manuscript.

**10. Table of Materials: Please sort the materials alphabetically by material name.**

The changes have been made accordingly in the revised version of the manuscript.

**11. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.**

The changes have been made accordingly in the revised version of the manuscript.

**12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below: Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).**

The changes have been made accordingly in the revised version of the manuscript.

#### **Reviewers' comments:**

##### **Comments Reviewer 1:**

We thank the reviewer for their valuable comments. Please find our responses below.

##### **Major Concerns:**

**The major concern is a lack of details. The authors should submit videos at least detailed pictures to indicate the surgery process. Also, the authors need to exclusively state the novelty of this method compared to previously reported methods. Without such details, it is hard to judge whether this work is acceptable by JOVE or not.**

In agreement with this suggestion, we have included pictures to provide details about the procedure in the revised version of the MS (please see Figures 1-14). It is important to note that “novelty is not a requirement for publication”. However, we have, for the first time, described a method for assessing cardiac function with an erythrocyte-perfused “working heart”-model in a video.

## **Comments Reviewer 2:**

We thank the reviewer for their valuable comments. The changes have been made accordingly in the revised version of the manuscript.

### **Major Concerns:**

**Line 132: May piritramide induce preconditioning and thus may affect infarct size?**

Analgesia is an important aspect thus we should follow the standard operation procedure in our animal studies (local and national). Previous studies have demonstrated that opioids reduce injury from myocardial ischemia-reperfusion in preclinical and clinical studies. In our experimental setting, we cannot exclude the infarct size-limiting effect of piritramide, however we used relative low dosage and our infarct (scar) formation was comparable with other studies (PMID: 30904281). Please note that we used piritramide for analgesia, not for anaesthesia. Moreover, a previous study (PMID: 4026787) showed the limiting effect of the infarct size by the anaesthesia (fentanyl) via a mechanism improving collateral flow. This latter effect was negligible in rodent models of acute myocardial ischemia.

### **Minor Concerns:**

**Line 148: not clear how the occlusion is performed for a reperfusion protocol.**

Ischemia was induced by tightening of the ligature around the left coronary artery. Successful occlusion was associated with ECG changes (ST-segment elevation) and macroscopic changes in the left ventricle. Reperfusion was initiated after 30 min of ischemia by removal of the snare. The reperfusion was associated with disappearance of the cyanotic colour of the myocardium.

**Size of the suture? – Please see Chapter 3- surgical procedure**

The size of the suture has been included in the revised version of MS (please refer to the list of materials).

**Fig 2: How long is the ischemia since the "infarct size" looks large**

Ischemia was induced by tightening of the ligature around the left coronary artery for 30 min. In our study, the primary end-point was to assess cardiac fibrosis, not the

infarct size. In addition, the area of fibrosis is comparable with findings in other small animal studies.

**Figure legends: what are the statistical analyses performed?**

We did not include a description of the statistical methods because the purpose of the presented results is to underline the efficacy of the procedures and to evaluate them. However, our results were presented as mean  $\pm$  SEM. One-way ANOVA followed by Tukey post-hoc tests were performed for comparing the LV hemodynamic parameters in vivo (Echo), ex vivo (WH apparatus. See in PMID: 30904281.

**Comments Reviewer 3:**

**1) There were no major or minor concerns.**

We thank the reviewer for their comment.

## **Revision No.2 of manuscript JoVE61033, Response to editor's comments**

### **Editorial comments:**

We want to thank the editor once again for his valuable comments. Please find our responses below.

- 1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.**

Thank you for these changes.

- 2. The protocol text should provide a detailed description to enable the accurate replication of the presented method (including setup, materials, actions, conditions, etc.) by both experts and researchers new to the field. For each protocol step, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please see specific comments marked in the attached manuscript.**

These have been changed in the revised version of the MS. In addition, we referred one of our previous MS, which described the working heart models in detail. (Kiss et al., PMID: 29547976)

- 3. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text. Please highlight complete sentences (not parts of sentences) and ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please do not highlight any steps describing anesthetization and euthanasia. Please note that I have highlighted ~2 pages of protocol for filming. Please review and make changes as needed.**

Thank you very much for marking. Small changes have been performed and important steps are selected. As requested, no anesthesia descriptions were highlighted.

- 4. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.**

This has been done accordingly (PDFs).

**5. Figure 1-Figure 10: Please reduce the dimensions of these figures and combine them into one multi-panel figure.**

This has been done accordingly.

Please see: Figures\_1-10\_JoVE

**6. Figure 11 and Figure 12: Please combine them into one figure.**

This has been done accordingly. Please see: Figures\_11-12\_JoVE

**7. Figure 13 and Figure 14: Please combine them into one figure.**

This has been done accordingly. Please see: Figures\_13-14\_JoVE

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Publisher	MyJove Corp.
Expected publication date	Feb 2020
Portions	Fig. 1 a-f, page 74 Fig. 2 a-g, page 75



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